Ultrastructure of the basal lamina of bovine ovarian follicles and its relationship to the membrana granulosa

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Different morphological phenotypes of follicular basal lamina and of membrana granulosa have been observed. Ten preantral follicles (< 0.1 mm), and 17 healthy and six atretic antral follicles (0.5–12 mm in diameter) were processed for light and electron microscopy to investigate the relationship between follicular basal lamina and membrana granulosa. Within each antral follicle, the shape of the basal cells of the membrana granulosa was uniform, and either rounded or columnar. There were equal proportions of follicles ≤ 4 mm in diameter with columnar basal cells and with rounded basal cells. Larger follicles had only rounded basal cells. Conventional basal laminae of a single layer adjacent to the basal granulosa cells were observed in healthy follicles at the preantral and antral stages. However, at the preantral stage, the conventional types of basal lamina were enlarged or even partially laminated. A second type of basal lamina, described as 'loopy', occurred in about half the preantral follicles and in half the antral follicles ≤ 4 mm diameter. 'Loopy' basal laminae were not observed in larger follicles. 'Loopy' basal laminae were composed of basal laminae aligning the basal surface of basal granulosa cells, but with additional layers or loops often branching from the innermost layer. Each loop was usually < 1 μm long and had vesicles (20–30 nm) attached to the inner aspect. Basal cellular processes were also common, and vesicles could be seen budding off from these processes. In antral follicles, conventional basal laminae occurred in follicles with rounded basal granulosa cells. Other follicles with columnar cells, and atretic follicles, had the 'loopy' basal lamina phenotype. Thus, follicles have different basal laminae that relate to the morphology of the membrana granulosa.

Introduction

The mammalian ovary contains a pool of inactive primordial follicles. Each follicle is composed of a small inactive oocyte and a single layer of non-replicating granulosa cells enveloped by a basal lamina. In adults, a few primordial follicles become activated each day and the oocyte begins to enlarge and the granulosa cells divide. Layers of granulosa cells form, and eventually a fluid-filled antrum develops in the middle of the follicle. These stages of follicle development are referred to as preantral and antral, respectively. Only large healthy antral follicles are capable of ovulating, and only a very small proportion of follicles will ever reach ovulatory size and ovulate their oocyte. Most ($> 99\%$) follicles become atretic and regress, in a process initially characterized by death of the granulosa cells.

Studies in many mammalian species of the dynamics of follicles growing in a pair of ovaries during the reproductive cycles have led to the concepts of follicle 'recruitment' and follicle 'dominance'. Follicle recruitment can refer to a group of follicles growing at the same time and at a similar rate, as if in a wave. Follicle dominance refers to one follicle growing

at a faster rate than other subordinate follicles, which eventually become atretic. If the dominant follicle is eliminated after atresia of the subordinate follicles has commenced, another cohort of growing follicles is recruited from the pool of follicles of smaller size. Unfortunately, the physiological regulation of recruitment and dominance is not well understood.

Two or three waves of follicles emerge in each bovine oestrous cycle. Follicles in each wave grow to about 5 mm in diameter before one dominant follicle emerges and enlarges to > 10 mm. Follicles of this diameter are sufficiently large to be able to ovulate, if simulated by a surge release of LH from the anterior pituitary gland. The structure of the membrana granulosa varies among antral follicles < 5 mm in diameter both in terms of the number of layers and in the shapes of the granulosa cells (van Wezel *et al.*, 1999). Some follicles have basal cells that are columnar in shape, with rounded cells in the middle layers and flattened cells aligning the antrum, while other antral follicles have rounded cells throughout the membrana granulosa.

In each follicle, the epithelioid membrana granulosa is enveloped by a follicular basal lamina that separates it from the surrounding stromal elements in primordial and

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preantral follicles (van Wezel and Rodgers, 1996) or from the specialized theca in antral follicles. The follicular basal lamina is believed to play a role in influencing granulosa cell proliferation and differentiation (Amsterdam *et al.*, 1989; Richardson *et al.*, 1992; Luck, 1994). In addition, in healthy follicles, the basal lamina excludes capillaries, blood cells and nerve processes from the membrana granulosa until ovulation, at which time it is degraded.

Considerable variation has also been observed among bovine follicles in the ultrastructure of the follicular basal lamina (Rodgers *et al.*, 1995; van Wezel and Rodgers, 1996). In the present study, the ultrastructure of the follicular basal lamina of preantral and antral follicles was characterized and, in healthy antral follicles, was found to be related to the structure of the membrana granulosa.

Materials and Methods

All bovine tissues were collected from cows assessed visually as being non-pregnant, within 20 min of being killed at a local abattoir. Eleven reproductive tracts were collected and one ovary per tract was perfusion fixed. Briefly, the ovarian artery was cannulated, and the associated ovary flushed with 50 ml Earle's balanced-salt solution (EBSS) and then 20 ml 2.5% (v/v) glutaraldehyde in 0.1 mol phosphate buffer l^{-1} (pH 7.25) at room temperature. Antral and preantral follicles with adhering stroma were dissected and placed in fresh 2.5% glutaraldehyde fixative to aid penetration of the fixative into the follicular fluid ($n = 11$ ovaries, 1-5 antral follicles examined per ovary). The diameter of the follicles was measured. Fixation of whole antral follicles caused the follicular fluid to gel. It was then possible to cut wedge-shaped blocks of fixed tissue containing follicular fluid, membrana granulosa, basal lamina, theca and stroma. The blocks of tissues were further fixed in glutaraldehyde and then post-fixed with 1% (v/v) osmium tetroxide, dehydrated in graded concentrations of acetone (v/v), and embedded in epoxy resin by standard methods. Thick sections (1 μm) were stained with 1% (w/v) methylene blue, examined using an Olympus BX50 microscope, with or without Nomarski optics, and photographed using an Olympus C35AD-4 camera attachment and Kodak T-Max 400 black and white film. Ultrathin sections were stained with uranyl acetate and lead citrate and observed and photographed with a JEOL CS1200 electron microscope (Peabody, MA).

Follicles were classified as preantral if they were small with more than one layer of granulosa cells surrounding an oocyte, and with no evidence of an antrum. No identifiable theca cells were present in these follicles. Antral follicles were classified as healthy if they had intact layers of granulosa cells, and no or very few pyknotic granulosa nuclei. Atretic follicles had many pyknotic nuclei. Severely regressing follicles were not examined in this study. All light microscopy was carried out by one examiner, who classified the follicles as healthy or atretic and further classified antral follicles as 'columnar' or 'rounded' on the basis of the shape of the basal granulosa cells (Fig. 1). The electron microscopic examination was made by a second examiner, without reference to the results of the classifications of the first examiner.

Results

Ten preantral follicles, and 17 healthy and six atretic antral follicles (0.5–12 mm) were examined. The basal laminae of antral follicles were either a conventional single layer, neatly aligned to the membrana granulosa, or they were composed of many layers or loops (Figs 2 and 3). In these 'loopy' basal laminae, a conventional basal lamina was aligned to the basal surface of the basal granulosa cells, but additional layers or loops often branched from the innermost layer

Fig. 1. Photomicrographs of 1 μm thick methylene blue-stained epoxy sections of bovine antral follicles exhibiting either (a) rounded or (b) columnar basal granulosa cells adjacent to the follicular basal lamina (arrowheads). (a) 10 mm follicle; (b) 1.5 mm follicle. Scale bars represent 10 μm .

Fig. 2. Electron micrographs of the bovine follicular basal laminae of antral follicles each showing a different phenotype. (a) The conventional basal lamina consists of a single layer closely abutting the adjacent granulosa cell layer (arrowheads). (b) The 'loopy' basal lamina is multilayered and not all layers are in close contact with the basal granulosa cells (arrows). (a) 4 mm follicle; (b) 1.5 mm $\times$ 0.8 mm follicle. Scale bars represent 1 μ m.

(Fig. 3b). Each loop was usually < 1 μ m long and had vesicles (20–30 nm) attached (Fig. 4) to the inner aspect (Figs 3b and 5b), occurring at about every 100–200 nm. Basal cellular projections (Figs 3b and 5a) were often observed on the basal granulosa cells in follicles with 'loopy' basal lamina, and vesicles could be seen budding off from these processes (Fig. 3b).

In initial studies, sections of follicles were cut in different planes to prove that the shape of the basal cells could not be apparently altered by merely cutting the block of a follicle wall in a different plane. Antral follicles were then classified as either 'columnar' or 'rounded' on the basis of the shape of the basal granulosa cells. Antral follicles with rounded basal cells were of all sizes. However, follicles with columnar basal

Fig. 3. High power electron micrographs of (a) conventional single-layered (arrowheads), and (b) 'loopy' multi-layered follicular basal laminae. Arrow denotes a vesicle in close contact with both the follicular basal lamina and the plasma membrane of a cellular projection. (a) 3.5 mm $\times$ 4.5 mm follicle; (b) 0.25 mm $\times$ 0.35 mm follicle. Scale bars represent 100 nm.

cells were only observed in follicles ≤ 4 mm in diameter. In this size category (≤ 4 mm), follicles of either columnar or rounded cells occurred in about equal proportions, as in a previous study of 80 antral follicles in which follicles with columnar cells were observed only in follicles < 5 mm (van Wezel *et al.*, 1999).

In healthy antral follicles, the type of follicular basal lamina correlated with the type of membrana granulosa. All healthy antral follicles with a 'loopy' basal lamina had a membrana granulosa that had columnar basal granulosa

cells (Fig. 1b), and all these follicles were ≤ 4 mm in diameter. Conversely, all antral follicles with conventional basal lamina had rounded basal granulosa cells (Fig. 1a; Table 1). Since these follicles were of all sizes, they were further subdivided into two groups, ≤ 4 mm or > 4 mm. The mean diameter of the ≤ 4 mm group was similar to that of the follicles with rounded basal cells (Table 1). Comparing these two groups of similar-sized follicles showed the same association between basal granulosa cell shape and the basal lamina phenotype (Table 1). Thus, the difference between the

Table 1. The proportion (number of follicles with each type of basal lamina per total numbers of follicles examined) of antral follicles with either columnar or rounded (divided into size categories ≤ 4 mm or > 4 mm) basal granulosa cells that have either a 'loopy' or 'conventional' basal lamina

Basal lamina type	Shape of basal granulosa cell		
	Columnar	Rounded	
	All ≤ 4 mm (2.3 ± 0.6 mm)*	≤ 4 mm (2.5 ± 0.5 mm)*	> 4 mm (11.0 ± 0.9 mm)*
Loopy	5/5	–	–
Conventional	–	6/6	6/6

*Mean $\pm$ SEM diameter.

Fig. 4. High power electron micrograph of a bovine 'loopy' basal lamina showing the membrane-bound matrix vesicles associated with the basal lamina. Scale bar represents 50 nm.

Fig. 5. Electron micrographs of bovine 'loopy' basal lamina, showing (a) cellular projection (arrowhead), and (b) the location of matrix vesicles (arrows) on the granulosa side of the loops. (a) 0.8 mm $\times$ 1.5 mm follicle; (b) 2.0 mm $\times$ 3.8 mm follicle. Scale bars represent 200 nm (a) and 100 nm (b).

follicles with rounded or columnar basal cells is clearly not simply a function of differing diameter.

In preantral follicles, basal laminae were also of two types, 'loopy' or 'conventional'. However, the conventional basal laminae of preantral follicles were often laminated (Fig. 6a) or at least thickened (Fig. 6b) compared with the conventional basal laminae observed in the antral follicles. Preantral follicles with 'loopy' basal laminae comprised four of the ten preantral follicles examined, whereas four of ten preantral follicles had a thickened basal lamina and the remainder had a basal lamina that was conventional with some loops.

Five of the six atretic antral follicles examined had a 'loopy' basal lamina, and the remaining one had regressed sufficiently not to have a distinctive basal lamina.

Discussion

In the present study, two different phenotypes of follicular basal lamina that alter with follicular development are described. Conventional basal laminae of a single layer can

Fig. 6. Electron micrographs of bovine basal laminae of preantral follicles (<0.1 mm) showing (a) a basal lamina of more than one layer (arrows) and (b) a thickened basal lamina. Scale bars represent 200 nm.

be found in healthy follicles at the preantral and antral stages. However, at the preantral stage, the conventional basal laminae were enlarged or even partially laminated. A second phenotype of 'loopy' basal lamina occurred in about half the preantral follicles and in half the antral follicles ≤ 4 mm in diameter. 'Loopy' basal laminae were not observed in larger follicles. In antral follicles the 'loopy' phenotype occurred in association with basal granulosa cells that were columnar in shape, whereas other follicles with rounded basal cells had a conventional basal lamina. Since it is not possible to track the development of individual follicles at the preantral stage, it has not previously been recognized that there are two clearly distinct phenotypes of follicles at the preantral stage.

In cows, there are two or three waves of follicular development during an oestrous cycle (Fortune, 1991;

Ginther *et al.*, 1996). Each wave consists of the contemporaneous appearance, which can be observed by ultrasonography, of 3–6 follicles ≥ 5 mm in diameter (ovulatory size is 10–20 mm). Growth up to this stage (4 mm) seems not to require stimulation by LH or FSH (Gong *et al.*, 1996). Several days after the emergence of a wave, one follicle becomes larger than the rest; it continues to grow while the smaller follicles in the cohort regress. In a non-ovulatory wave, the dominant follicle remains as the largest follicle for several days even though the succeeding wave has commenced. Thus, a follicle may retain size dominance longer than it retains functional dominance (Fortune, 1991). The corollary of these dynamic changes in follicle growth is that healthy antral follicles are clearly of two types, dominant and non-dominant, characterized by whether they are actively growing or growing only slowly or not at all.

Thus, it was not surprising to find different phenotypes of the follicular basal lamina and membrana granulosa.

The significance of the shape of basal granulosa cells has been discussed in other species. Hirshfield (1991) has suggested that, shortly after antrum formation in rats, some of the mural granulosa cells withdraw from the cell cycle, losing their cuboidal shape and assuming a columnar appearance. In sheep, Gougeon (1996) has reported from the data of Jablonska-Shariff *et al.* (1994) that columnar basal granulosa cells exhibited a greater rate of proliferation than did more antrally situated granulosa cells. However, despite these suggestions, the significance of rounded cells and columnar granulosa cells is not clear. The shape of the basal granulosa cells may reflect the dynamics of the follicle as a whole, rather than individual differences between the cells within the membrana granulosa.

During follicular development there are, on average, a net 19 doublings in the surface area of the follicle (from a primordial to an 18 mm bovine follicle, calculated from van Wezel and Rodgers, 1996), and a net 21 doublings of the number of granulosa cells (if 40×10^6 granulosa cells were present, as reported by McNatty *et al.*, 1984; see van Wezel and Rodgers, 1996). Thus, it is predicted from these calculations that cell layers in the membrana granulosa would increase from one layer, as in primordial follicles, to four layers, since the increase in the number of cells is four times that of the increase in surface area (calculated as $(21-19)^2$) (R. J. Rodgers *et al.*, 1998, 1999). Considerable variation in the number of layers per follicle has been reported (van Wezel *et al.*, 1999), consistent with a reported variation in the number of granulosa cells obtained from follicles of the same size (McNatty *et al.*, 1979). On the basis of these observations, it has been suggested that the rate of granulosa cell proliferation and maturation is not tightly or co-ordinately regulated with the rate of antrum expansion (R. J. Rodgers *et al.*, 1998, 1999).

Both the laminin (van Wezel *et al.*, 1998) and type IV collagen (H. F. Rodgers *et al.*, 1998) composition of the follicular basal laminae change during follicular development. The follicular basal lamina probably has a role retarding the entry of larger molecular weight plasma proteins and molecules (for example, low density lipoproteins) into the follicular antrum (Andersen *et al.*, 1976). The molecular weight cut-off in antral follicles has been calculated to be 100–850 kDa on the basis of a comparison of the composition of follicular fluid with that of plasma (Andersen *et al.*, 1976). Size may not be the only determinant of movement across the follicular basal lamina. It has been shown that the blood–follicle barrier to movement of inter- α -inhibitor is due to its negative charge (Hess *et al.*, 1998), indicating that the follicular basal lamina may also exclude material on this basis. As yet, it has not been determined whether the size or type of material able to cross the follicular basal lamina changes during follicular development, especially during antrum formation and expansion. However, in other organs, different forms of basal lamina have been observed and related to tissue development or disease states. In both insulin- and non-insulin-dependent diabetics, many basal laminae are composed of many layers or of one thick layer (Martinez-

Hernandez and Amenta, 1983; Abrahamson, 1986). In the diabetic kidney, the glomerular basal lamina is considerably expanded, of different composition, and more permeable to serum proteins (Martinez-Hernandez and Amenta, 1983; Abrahamson, 1986). In the present study, the conventional basal lamina in preantral follicles was considerably thicker, or laminated, compared with the conventional basal lamina of antral follicles. These differences in the structure of the conventional basal lamina between preantral and antral follicles may reflect changes in its permeability.

The appearance of the 'loopy' follicular basal lamina is consistent with it being shed and replaced by new basal lamina near to the basal surface of the basal granulosa cells. Cellular projections were observed emanating from the basal surface of the basal granulosa cells and vesicles, often at the end of these processes next to the basal lamina. Matrix vesicles were often attached to the basal lamina on the side facing the granulosa cells. These observations are consistent with the suggestion that the loops of basal lamina were once adjacent to the cell, and were shed from the cellular surface as the cells developed processes and then released matrix vesicles. Murata *et al.* (1994) have observed the folding of the basal lamina of the small intestine of tadpoles during metamorphosis and hypothesized that, as the small intestine shortened, the resulting rapid loss of space caused folding of the remaining basal lamina, which was in excess. If the 'loopy' basal lamina was formed as the result of a similar tissue remodelling process, this implies that the follicles with 'loopy' basal laminae, which have been shown also to have columnar basal cells, are slow growing or even shrinking.

Matrix vesicles are right-side-out vesicles of cellular origin and have been well characterized in bone, cartilage and dentine tissues (Anderson, 1995). In skeletal tissues, matrix vesicles (30 nm to 1 μ m in diameter, mean approximately 200 nm) usually arise by the budding and pinching-off of specific regions of the outer cell membrane. The conditions that elicit budding of matrix vesicles are unknown, but are presumed to be related to the cell cycle and possibly to the process of apoptosis (Anderson, 1995). Lee *et al.* (1993) described the cellular ectocytotic release of 200 nm matrix vesicles selectively derived from the cell surface of fibroblasts during stress-relaxation. These authors suggested that fibroblast matrix vesicles play a role in the tissue remodelling that begins soon after the contraction phase of wound repair. Vesicles have also been observed associated with other basal laminae. The thick basal lamina of the tubular portion of the nephron of the one-humped camel has been observed to contain 10–500 nm extracellular membrane-bound bodies of various degrees of electron opacity, with or without 5–15 nm intramembranous particles. These basal lamina-associated bodies may play a role in the ionic transport mechanisms associated with the high osmolarity of camel urine (Safer and Katchburian, 1991). Vesicles have also been observed associated with basal laminae secreted by granulosa cells in anchorage-independent culture (Rodgers *et al.*, 1995). The true role of matrix vesicles in basal laminae is not clear at this stage. However, given their association with different phenotypes of membrana granulosa, and not with follicles that have reached the size of dominance, the suggestion that matrix

vesicles in basal laminae are involved in tissue remodelling is not unreasonable.

In summary, in preantral follicles, both 'loopy' and conventional, albeit enlarged, basal laminae were observed. This finding is of great interest for the study of follicle dynamics, as it indicates that not all preantral follicles are alike. A study of the structure of the membrana granulosa of preantral follicles has yet to be made, and so it is not known whether the two phenotypes of basal lamina are, as in antral follicles, related to features of the membrana granulosa. While the significance of these phenotypes of membrana granulosa and basal lamina have been speculated upon, further investigation is necessary.

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